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in Neurodevelopment

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14. ABSTRACT An immunopathology reported in some autistic patients is development of autoantibodies against brain-specific proteins, which suggests impacts to regulatory T cells (Tregs). Perfluorooctanoic acid (PFOA) and perfluorooctane sultamate (PFOS) are widespread environmental pollutants and induce developmental and immuno-toxicity. Our hypothesis is that developmental exposure to PFOA or PFOS will affect number and/or function of Tregs and increase autoimmune risk in offspring. In immunocompetent male and female offspring exposed to PFOA or PFOS during gestation and lactation, splenic Treg number and function, serum markers of autoreactivity, and levels of myelin basic protein and T cell infiltration in the cerebella were evaluated. Treg ex vivo function was decreased in male offspring at all doses of PFOA and Treg numbers were decreased in female offspring exposed to 2 mg/kg of PFOA. These data suggest that the functional capacity of Tregs may be undermined by developmental exposure to PFOA. Additional analysis will determine if PFOS induces similar effects.					
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INTRODUCTION

The etiology of autism and related neurodevelopmental disorders is largely unknown. Myriad hypotheses have suggested that exogenous agents, such as environmental pollutants, play a role in causing or triggering dysfunctional development that may culminate in an autism diagnosis. It also has been suggested that immunopathogenesis, or alterations to the immune system, occur in subsets of autistic patients. One type of immunopathology that has been reported in some autistic patients is the development of autoantibodies against brain-specific proteins, which suggests that regulatory T cells (Tregs) or central/peripheral tolerance (the process by which autoreactive T cells are isolated or eliminated) may be impacted. The emerging contaminants perfluorooctanoic acid (PFOA) and perfluorooctane sulfonate (PFOS) are environmentally pervasive and have been associated with both developmental toxicity and immunotoxicity. It is therefore plausible that exposure to these compounds may affect Tregs or central/peripheral tolerance and may result in dysregulation of autoreactive T cells and subsequent neural damage. Our hypothesis is that developmental exposure to PFOA or PFOS will affect the number and/or function of regulatory T cells (Tregs) and lead to changes indicative of increased autoimmune risk in offspring. In immunocompetent male and female offspring that were exposed to PFOA or PFOS during gestation and lactation, we evaluated splenic Treg number and function, serum levels of anti-MBP, anti-dsDNA, and anti-ssDNA, and levels of myelin basic protein and T cell infiltration in the cerebella. The findings of our studies will establish whether PFOA and/or PFOS impact Tregs and/or tolerance during development and create a system that may lead to autoimmune reactions against neural tissues.

BODY

Specific Aim #1: To determine the effects of developmental exposure to perfluorooctanoic acid (PFOA) or perfluorooctane sulfonate (PFOS) on regulatory T cells (Tregs; CD4+CD25+FoxP3+) and autoantibody production in C57Bl/6 mice by assessing Treg number with flow cytometry, Treg function by evaluation of IL-10 and perforin* secretion ex vivo, and markers of autoreactivity with serum anti-MBP (myelin basic protein), anti-ssDNA, and anti-dsDNA.

*Note: perforin was measured by flow cytometry from cells cultured ex vivo. However, perforin levels in cultured Treg cells were too low to be detected. Both perforin and granzyme B are secreted by Tregs to induce cell death of host immune cells. However, although we cultured Tregs with IL-2, we believe that they were not sufficiently activated to produce a level of perforin in culture that we could detect with flow cytometry. Alternatively, low levels of perforin in cultured Tregs could indicate insufficient function of Tregs induced by developmental PFOA or PFOS exposure. Additional research is required to verify such functional defects.

Tasks 1 and 2. Developmental effects of PFOA exposure in C57Bl/6 mice. All studies completed. Data analysis completed. Manuscript write-up ongoing.

Tasks 3 and 4. Developmental effects of PFOS exposure in C57Bl/6 mice. All studies completed. Data analysis ongoing. Manuscript write-up ongoing.

Results of Tasks 1 and 2

In two replicate experiments (DoD-1 and DoD-2), 96 dams were gavaged with PFOA from pairing with males through weaning of pups, which exposed offspring from gestational day zero (GD0) through weaning, at PND21. The following endpoints were collected in subsets of the adult offspring:

- Number of splenic Tregs (CD4+CD25+FoxP3+) as determined by flow cytometry
- Ex vivo Treg (CD4+CD25+FoxP3+) function as measured by IL-10 release
- Serum markers of autoreactivity (anti-dsDNA, anti-ssDNA, and anti-MBP)

Reproductive and developmental outcomes

Pregnancy success was 67%, 46%, 54%, and 58% for the 0 mg/kg, 0.02 mg/kg, 0.2 mg/kg, and 2 mg/kg dose groups, respectively (Table 1). These success rates were not statistically different from one another ($P < 0.05$) and are appropriate for the C57Bl/6 strain of mouse. Number of litters delivered and number of litters weaned (Table 1) did not differ statistically ($P < 0.05$) by dose, which indicates that the administered PFOA doses were not overtly fetally toxic.

Table 1. Reproductive outcome of dams dosed with PFOA via gavage from pairing with males through weaning of pups and litter endpoints. DoD-1 and DoD-2 studies combined. N = 24 dams/dose group.

	0 mg/kg PFOA	0.02 mg/kg PFOA	0.2 mg/kg PFOA	2 mg/kg PFOA
Dams pregnant	16	11	13	14
Litters delivered	13	11	13	14
Litters weaned	7	9	11	9
# of litters for Treg #	7	8	11	7
# of litters for Treg function	6	7	9	6
# of litters for autoreactivity	3	2	4	4

Developmental exposure to PFOA did affect the weight of litters (Figure 1). Litters from the dams exposed to 2 mg/kg of PFOA had statistically lower body weights ($P < 0.05$) throughout the lactational period, from birth at postnatal day (PND) one through weaning at PND21. Litter weights in the 2 mg/kg dose were reduced relative to litter weights in the control group by 17.3%, 49.8%, 30.6%, and 32.7% at PND1, PND9, PND16, and PND21, respectively. Although we did not monitor developmental milestones in the study, anecdotally, we noticed that pups from these litters were visibly runted and developed hair and opened eyes at a later age compared to other dose groups and the control group. Five of the nine litters from this group were weaned at 23-27 days of age rather than 21 days of age due to delays in development.

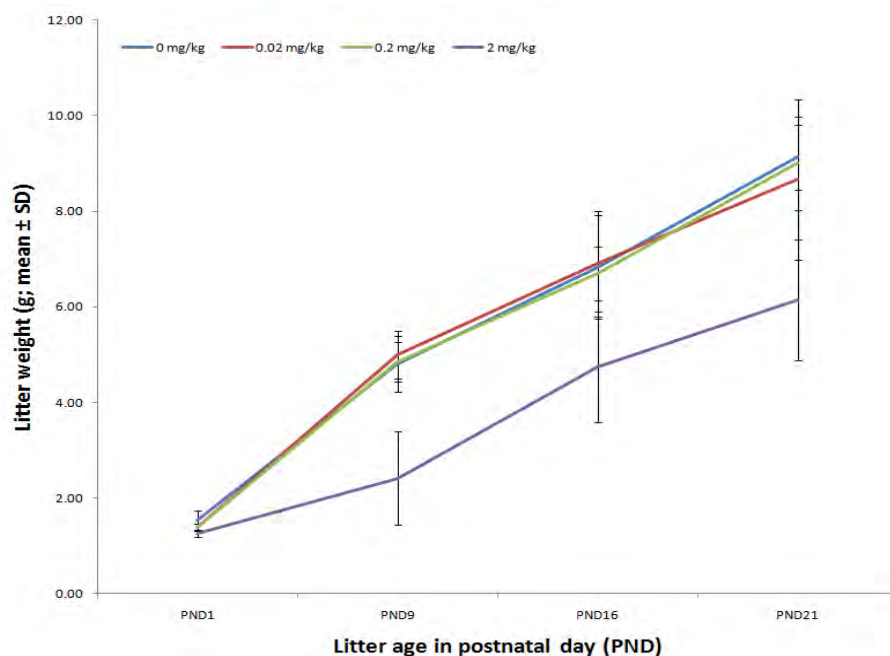


Figure 1. Mean litter weights (\pm standard deviation) of offspring from dams dosed with PFOA via gavage from pairing with males through weaning of pups. Mean litter weights of offspring in the 2 mg/kg dose group was statistically ($P < 0.05$) lower from all other dose groups at all time points.

Immunophenotyping

The number of splenic CD4+CD25+Foxp3+ Tregs were counted in subsets of offspring (Figure 2). Single cell suspensions were made from spleens of individual animals, stained for CD4+, CD25+, and Foxp3+, and counted in an Accuri flow cytometer. The resulting percentage of cells positive for CD4, CD25, and Foxp3 were determined from 25,000 events (cells). The percent of T cells with this phenotype in the control group was approximately 8%, which was expected for this subset of T cells in the spleen. The offspring of the treated dams had slightly lower Treg numbers relative to offspring from control dams; however, only females from the 2 mg/kg dose had statistically lower numbers of Tregs (5.9%; $P < 0.05$).

Ex vivo Treg function

The ability of splenic CD4+CD25+ Tregs to release IL-10 in culture with target cells was determined from subsets of offspring. Single cell suspensions were made from pooled spleens of five representative males and five representative females from each dose group. If possible, offspring from the same dams were not pooled unless it was not possible due to low litter numbers for a particular dose. Briefly, CD4+CD25+ Tregs were isolated from pooled spleen cells and co-cultured ex vivo for 72 hours with CD4+CD25- T cells as targets, and stimulated with anti-CD3+, anti-CD28+, and IL-2 in RPMI culture medium. IL-10 levels were measured in the culture medium with an R&D Systems ELISA kit. Results are shown in Figure 3. IL-10 release from male offspring was decreased by 61%, 75%, and 75%, in the 0.02 mg/kg, 0.2 mg/kg, and 2 mg/kg dose groups, respectively ($P < 0.05$). In female offspring, levels of IL-10 released were increased by 204% in the 0.02 mg/kg dose group relative to controls ($P < 0.05$); no other differences were detectable in the female offspring.

Serum markers of autoreactivity

Serum markers of autoreactivity were measured in subsets of offspring. Serum markers included levels of anti-dsDNA, anti-ssDNA, and anti-myelin basic protein (anti-MBP). Figure 4 is a representative illustration of the results of the three different assays. No differences in autoreactivity markers were detected among dose groups or between sexes.

Summary of findings for Tasks 1 and 2 of Specific Aim #1

Litter weights of offspring exposed to 2 mg/kg of PFOA were reduced by 32.8%, on average. Treg numbers of female offspring exposed to 2 mg/kg of PFOA were reduced by 5.9%. Male C57Bl/6 mice given PFOA from gestation through lactation via exposure to dams exhibited a statistically significant decrease in the amount of IL-10 released from splenic Tregs cultured ex vivo. No other endpoints were statistically different by dose.

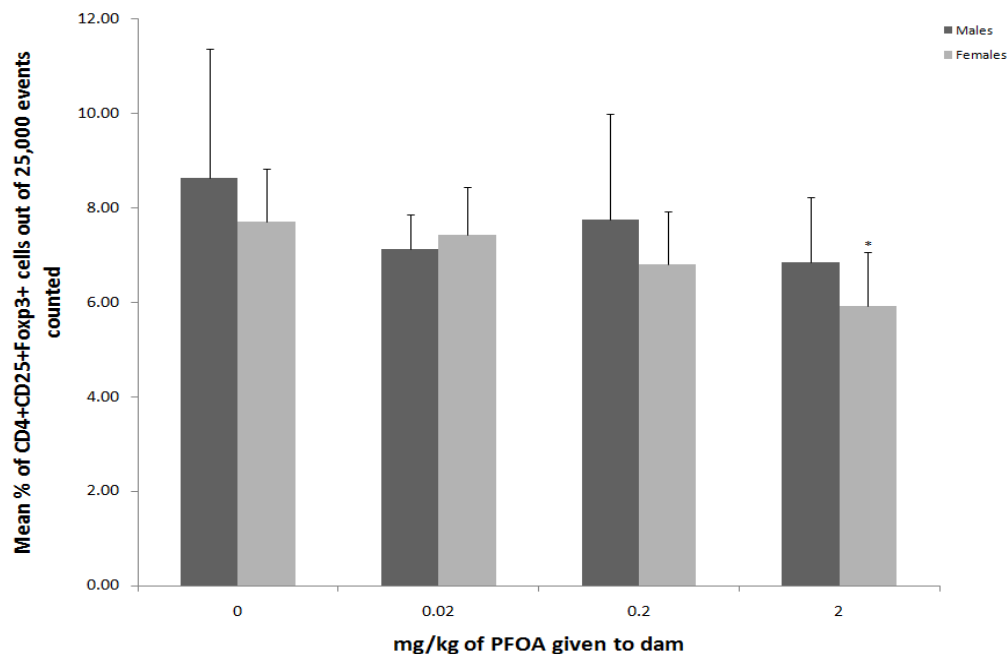


Figure 2. Mean percent (\pm standard deviation) of CD4+CD25+Foxp3+ regulatory T cells (Tregs) in offspring from dams dosed with PFOA via gavage from pairing with males through weaning of pups. * indicates statistical difference from same-sex control group ($P < 0.05$).

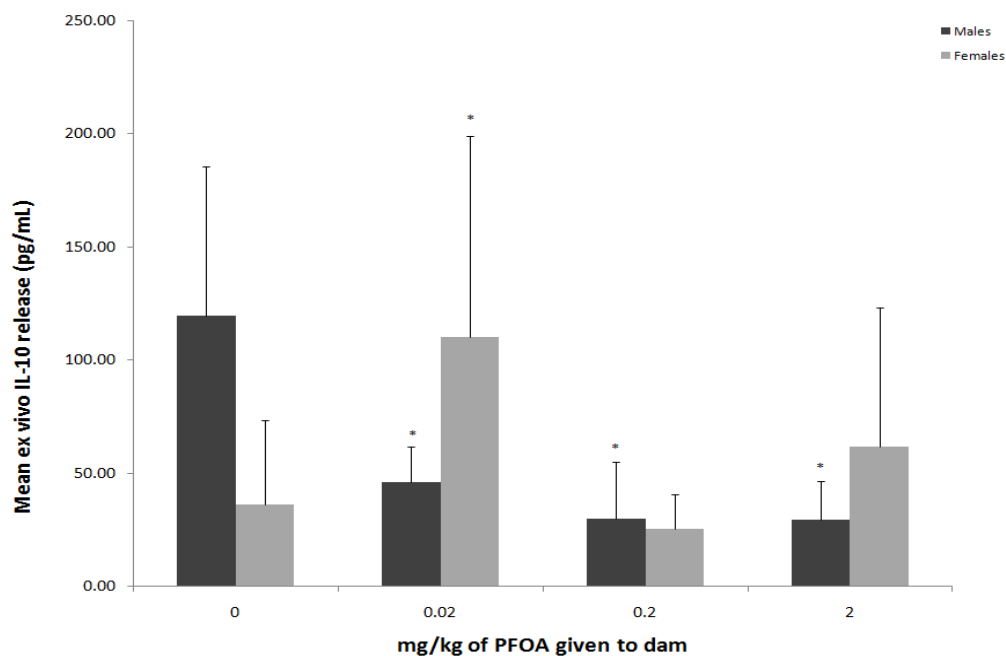


Figure 3A. Mean IL-10 (\pm standard deviation) released (pg/mL) from regulatory T cells (Tregs; CD4+CD25+Foxp3+) isolated from male offspring of dams dosed with PFOA via gavage from pairing with males through weaning of pups. Tregs were co-cultured ex vivo for 72 hours with CD4+CD25- T cells and stimulated with anti-CD3+, anti-CD28+, and IL-2 in RPMI culture medium.

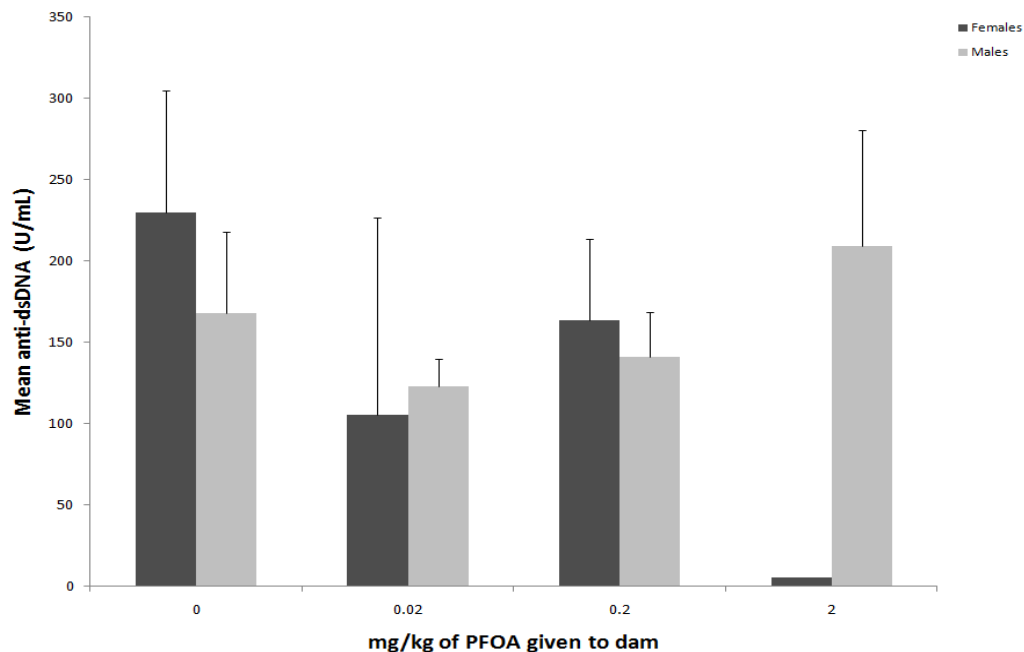


Figure 4. Mean serum (\pm standard deviation) anti-dsDNA of offspring from dams dosed with PFOA via gavage from pairing with males through weaning of pups. Levels of anti-dsDNA did not differ statistically by dose. Note: N = 1 for the female 2 mg/kg dose.

Specific Aim #2: To determine the effects of developmental exposure to perfluorooctanoic acid (PFOA) or perfluorooctane sulfonate (PFOS) on infiltration of T cells into the brains of C57Bl/6 mice by staining brain cerebellar sections with anti-CD3 and anti-MBP (myelin basic protein).

Tasks 1 and 2. Developmental effects of PFOA exposure in C57Bl/6 mice. All studies completed. Data analysis completed. Manuscript write-up ongoing.

Tasks 3 and 4. Developmental effects of PFOS exposure in C57Bl/6 mice. All studies completed. Data analysis ongoing. Manuscript write-up ongoing.

Results of Tasks 1 and 2

In two replicate experiments (DoD-1 and DoD-2), 96 dams were gavaged with PFOA from pairing with males through weaning of pups, which exposed offspring from gestational day zero (GD0) through weaning, at PND21. The following endpoints were collected in subsets of the adult offspring:

- Measurement of T cell infiltration into cerebella of exposed offspring
- Measurement of levels of myelin basic protein (MBP) in cerebella of exposed offspring

T cell infiltration into cerebella of exposed offspring

Numbers of CD3+ T cells that had infiltrated brains were counted in cerebella of subsets of offspring. Briefly, cerebella were immersion fixed in 10% neutral buffered formalin for 24 hours, paraffin-embedded, and sliced at 6 μ m with a rotary microtome. Sections were stained immunohistochemically with anti-CD3+ antibody and the number of cells stained with CD3+ were counted. A representative section is displayed in Figure 5. No CD3+ T cell infiltration was observed in any of the examined sections.

Levels of myelin basic protein (MBP) in cerebella of exposed offspring

Relative MBP levels were measured in cerebella of subsets of offspring. Cerebella were prepared as described above, but were stained immunohistochemically with anti-MBP antibody rather than anti-CD3+. The relative levels of MBP were determined and scored as normal, mildly depleted, moderately depleted, or severely depleted. The level of depletion observed in male and female brains is shown in Figure 6 and representative sections illustrating each level of depletion are shown in Figures 7A-7D. The average level of MBP depletion did not differ by dose.

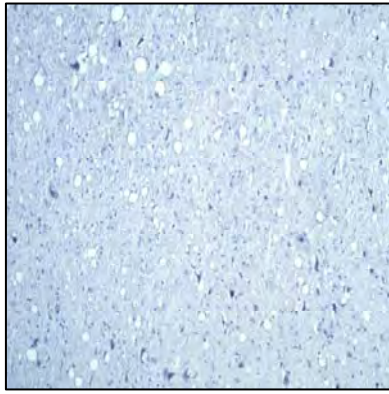


Figure 5. A representative cerebellar section from a female offspring of a dam dosed with PFOA via gavage from pairing with males through weaning of pups. No CD3+ T cell infiltration was observed in any of the examined sections (magnification =

Summary of findings for Tasks 1 and 2 of Specific Aim #2

Brains of C57Bl/6 mice given PFOA from gestation through lactation via exposure to dams did not have T cell infiltration or depletion of levels of MBP. No other endpoints were statistically different by dose.

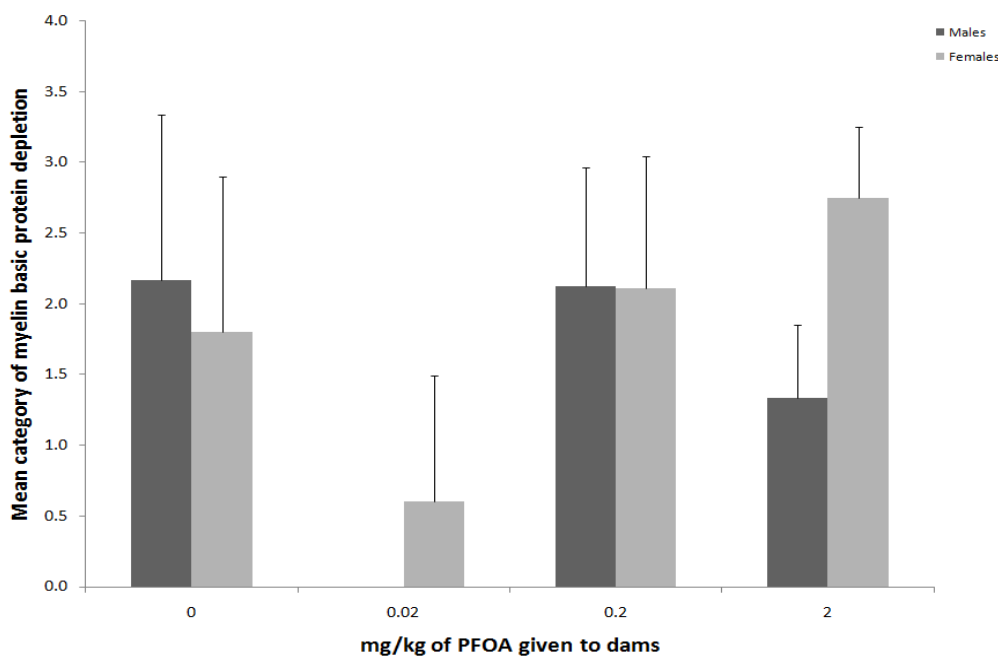


Figure 6. Mean category (\pm standard deviation) of myelin basic protein (MBP) depletion in cerebella of offspring from dams dosed with PFOA via gavage from pairing with males through weaning of pups. MBP depletion did not differ statistically by dose. Note: cerebella of males from the 0.02 mg/kg dose group were damaged artifactually and could not be read.

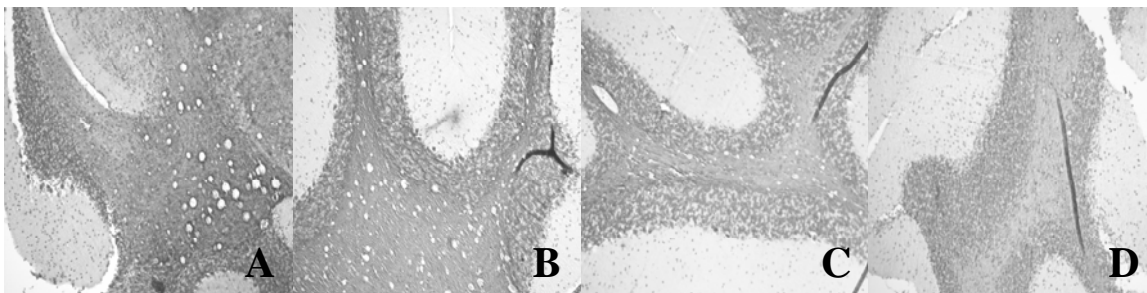


Figure 7. Representative cerebellar sections stained for myelin basic protein (MBP). A. Normal staining for MBP (male, 0 mg/kg, score = 0); B. Mild depletion of MBP (female, 0 mg/kg, score = 1); C. Moderate depletion of MBP (female, 0.02 mg/kg, score = 2); D. Severe depletion of MBP (male, 2 mg/kg, score = 3). Magnification = 20X.

KEY RESEARCH ACCOMPLISHMENTS

- Four developmental studies completed, two with perfluorooctanoic acid (PFOA) and two with perfluorooctane sulfonate (PFOS), and each involving exposure to 48 dams (192 dams total).
- Endpoints were assessed in 431 offspring.
- Numbers of CD4+CD25+Foxp3+ regulatory T cells (Tregs) in spleens of exposed offspring were successfully counted with a flow cytometer.
- Ex vivo function of CD4+CD25+ Tregs cultured from spleens of exposed offspring was successfully evaluated with IL-10 release.
- Serum markers of autoreactivity, including anti-dsDNA, anti-ssDNA, and anti-myelin basic protein (MBP) were successfully measured.
- Numbers of CD3+ T cells that had infiltrated brains of exposed offspring were successfully counted in cerebellar sections.
- The level of MBP depletion was successfully evaluated in cerebellar sections of exposed offspring.

REPORTABLE OUTCOMES

- Two manuscripts are in preparation for submission to *Toxicological Sciences*.
- Two high school students through the Pitt County Schools Honors Medicine Program presented findings on T cell infiltration and myelin basic protein levels in brains of offspring exposed to perfluorooctanoic acid (PFOA).
- One high school student through the Summer Ventures Program presented findings on T cell infiltration in brains of offspring exposed to perfluorooctane sulfonate (PFOS).
- One undergraduate student will present findings on T cell infiltration and myelin basic protein levels in brains of offspring exposed to perfluorooctane sulfonate (PFOS) at the annual NeuroToxicology Conference.
- The principle investigator, research technician, and graduate student will present whole study findings at the annual Society of Toxicology conference.
- A National Institutes of Health R21 grant will be resubmitted in November, 2011. Revisions to the application will be based on findings from the research accomplished through this grant project.

CONCLUSIONS

Specific Aim #1: To determine the effects of developmental exposure to perfluorooctanoic acid (PFOA) or perfluorooctane sulfonate (PFOS) on regulatory T cells (Tregs; CD4+CD25+FoxP3+) and autoantibody production in C57Bl/6 mice by assessing Treg number with flow cytometry, Treg function by evaluation of IL-10 and perforin secretion ex vivo, and markers of autoreactivity with serum anti-MBP (myelin basic protein), anti-ssDNA, and anti-dsDNA.

Specific Aim #2: To determine the effects of developmental exposure to perfluorooctanoic acid (PFOA) or perfluorooctane sulfonate (PFOS) on infiltration of T cells into the brains of C57Bl/6 mice by staining brain cerebellar sections with anti-CD3 and anti-MBP (myelin basic protein).

Conclusions of experiments for Specific Aims #1 and #2 (PFOA exposures only)

Little is known about how regulatory T cells (Tregs) impact normal neural development or how disruptions to Tregs by environmental contaminants may alter neural development. Tregs may be part of a regulatory process that tips the balance toward disease or health. In this project, female C57Bl/6 mice were given perfluorooctanoic acid (PFOA) from pairing with males through weaning of offspring to ensure that offspring were exposed throughout gestation and lactation. Endpoints assessed included: direct counts of splenic Treg numbers; direct evaluation of the ability of splenic Tregs to release suppressive cytokines by measuring ex vivo concentrations of IL-10; indirect evaluation of the ability of splenic Tregs to control autoreactive antibodies by measuring serum autoantibodies, T cell infiltration into brains, and levels of MBP in brains.

Effects of gestational and lactational exposure to 0.02 mg/kg, 0.2 mg/kg, or 2 mg/kg of PFOA resulted in the following statistically significant ($P < 0.05$) changes:

- A reduction in litter weights by 32.8%, on average, in offspring exposed to 2 mg/kg of PFOA.
- A reduction (5.9%) in Treg numbers in female offspring exposed to 2 mg/kg of PFOA.
- A 70% reduction, on average, of ex vivo IL-10 release from splenic Tregs cultured from male offspring exposed to all doses of PFOA.

Implications of these findings

The most important finding of our research is that developmental exposure to PFOA reduces the ability of cultured Tregs to release IL-10 in male offspring. Although the number of Tregs was slightly (5.9%) decreased in female offspring exposed to 2 mg/kg of PFOA, additional studies are required to determine if this change is biologically significant. IL-10 is a cytokine that modulates proinflammatory responses and when released by Tregs, may help to reduce secondary injury associated with inflammation during pathogen clearance (Maynard et al., 2007). Although our study was not designed to assess inflammatory pathways, a reduction in the ability of Tregs to release IL-10 suggests an impaired functional capacity that may extend to other suppressive functions of Tregs. Animal models that lack Tregs (i.e., scurfy mice) develop and succumb to autoimmune disease shortly after being weaned and autoimmune disease in humans is thought to arise, in part, by alterations to Tregs or to tolerance, which is a process by which unresponsiveness to self-antigens is developed. Reports of subsets of autistic patients that have increases in brain-specific autoantibodies (Silva et al., 2004; Wills et al., 2009) suggest that alterations to Tregs or the development of tolerance may contribute to an autistic phenotype. In addition, numerous studies have suggested that autism may arise from exposure to environmental agents that alter developmental processes. Our data demonstrate a reduced ability of ex vivo CD4+CD25+ Tregs to release IL-10, which indicates that the functional capacity of Tregs may be undermined by developmental exposure to PFOA, a widespread emerging environmental contaminant.

Recommended changes to future work

Autism likely arises from causative events that increase the risk to triggering events. A cause without a trigger and a trigger without a cause would not lead to an autistic phenotype. Developmental exposure to PFOA may be a sufficient causative event, but because we did not apply a potential trigger, we were not able to induce changes to brain morphology. Several studies have hypothesized that post-natal events, such as exposure to pathogens or environmental agents, trigger underlying immune dysfunction induced by other agents during gestational development (summarized in Dietert and Dietert, 2008). In a repeat study of this experiment, we would expose the offspring to a trigger, such as a influenza virus, during the lactation period. In this model, we would have both a potential causative agent and a post-natal trigger. In addition to evaluating the endpoints that we examined in the current study, we would include other endpoints such as behavior or neurochemistry

as these measures may be more sensitive than brain morphology. We also would evaluate the level of activated microglia in the brains of the offspring as microglia may be the immune cell in the brain that responds to immune alterations in the periphery.

So what do our results mean?

We cannot make any definitive conclusions about our entire project until we finish analyzing the data for the PFOS experiments (Tasks 3 and 4 of Specific Aims #1 and #2). The results of the PFOA studies suggest that the functional capacity of Tregs is affected by developmental exposure in male offspring. Our data have laid the groundwork for additional studies with Tregs and brain development.

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